10/539447

JC20 Rec'd PCT/PTO 2 0 JUN 2005

SYNTHESIS AND SREENING OF LIGANDS USING X-RAY CRYSTALLOGRAPHY

This invention relates to methods for synthesising compounds and
identifying from those compounds ligands that bind target

5   macromolecules using X-ray crystallography.

Background
Determining the structure of proteins by X-ray crystallography is
an elegant and reliable method and is the basis of structure-

10   based ligand design in which small molecules are synthesized as
potential ligands for the protein of interest. This is an intense
area of research for the optimisation of ligands to drugs for
therapeutically interesting proteins (see Babine, R.E. and
Bender, S.L., *Chemical Reviews*, **97**, 1359-1472 (1997) and Bohacek,

15   R.S., *et al.*, *Med. Res. Rev.*, **16**, 3-50 (1996)).

One method of ligand screening is described in WO 99/45379, in
which a library of shape-diverse compounds thought to be
potential ligands are soaked or co-crystallised with a target

20   protein, and then the resulting complex is analysed by X-ray
crystallography to determine the nature of the ligand which has
bound. The library of compounds which is used in the screening
process generally comprises previously characterised compounds.

25   Summary of the Invention
The above described method requires every potential ligand to be
synthesized, purified and characterized before it can form part
of a library for screening. If the potential ligands are simply
purchased from commercial sources, their costs will typically be

30   high due to the amount of work required to carry out these three
steps. If, in the alternative, the compounds are to be
synthesized 'in-house', then a great deal of time and effort will
need to be expended on assembling the library of ligands for
screening.   ,

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The present inventors have developed a method where a collection
of compounds is synthesized and then screened without the need

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for any purification and/or characterisation steps.

Accordingly, the present invention provides a method for identifying a ligand of a target macromolecule comprising the steps of:

5   steps of:

a) soaking one or more crystals of the target macromolecule in a solution containing a collection of compounds generated in situ or separate from the crystal, where the solution has been prepared without the purification, and

10   preferably without the characterisation, of the synthesized collection of compounds;

b) obtaining an X-ray crystal diffraction pattern of the soaked macromolecule crystal; and

c) using said X-ray crystal diffraction pattern to identify

15   any compound bound to the macromolecule crystal, said compound being a ligand of the target macromolecule.

Any solvated crystal system in which the solvent and/or ligand molecules are able to infiltrate throughout the crystal via

20   diffusion, and where the crystal system is compatible with X-ray diffraction data collection, is suitable for use in the invention.

Examples of appropriate macromolecules are polypeptides

25   (proteins), ribose nucleic acids (RNAs, ribozymes etc), deoxy ribose nucleic acids (DNAs), and complexes of combinations of the three examples, e.g. ribosomes, or viruses (DNA and/or RNA-protein complexes).

30   A ligand is a molecule which can bind to a macromolecule. For a polypeptide chain (protein), this is anything that is not coded for by the DNA sequence of the protein. This covers the post-translational modification of proteins (e.g. covalent attachment of sugars, etc.), the covalent and non-covalent attachment of

35   cofactors (e.g. Haem groups), the binding of other polypeptides or amino acids, the binding of small molecules (e.g. drugs, substrates, etc.) and the binding of DNA and RNA to proteins. For

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nucleic acids this is molecules either covalently, or non-
covalently bound to DNA or RNA (e.g. ligands intercalated between
bases).

5   In the present invention, the compounds can be synthesised by
     parallel synthesis, or (more conveniently) by combinatorial
     chemistry. Traditionally, combinatorial chemistry is used to
     generate small molecule inhibitors for screening against one or
     more biological targets. The synthesis of libraries of compounds
10   has generally been aimed at producing either compounds as
     purified single entities or as high-quality mixtures of compounds
     using methodology that allows for deconvolution of the mixture,
     once it has been determined that an active compound is to be
     found in that mixture. Deconvolution requires the re-testing of
15   each member compound of the active library. Thus either method
     requires the careful analysis and characterisation of the
     libraries to allow for interpretation of the data generated
     during biological screening against the target macromolecule. As
     mentioned above, the present invention does not require the.
20   purification and/or characterisation of the members of the
     synthesised library, as the identity of the ligand is determined
     by X-ray crystallography of the ligand-macromolecule complex.

25   The solution containing the collection of compounds can be
     prepared in two main ways, herein called 'in-situ' synthesis and
     'just-in-time' synthesis.

     'In-situ' synthesis involves synthesizing the collection of
30   compounds in a solution which also contains the one or more
     crystals of the target macromolecule, and therefore requires the
     use of chemistries which can be carried out under conditions in
     which the macromolecule crystal will remain stable.

35   Accordingly, a first aspect of the present invention provides a
     method for identifying a ligand of a target macromolecule
     comprising the steps of:

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a) synthesizing a collection of compounds, which are suitable for screening against a target macromolecule, in a solution containing one or more crystals of the target macromolecule;

5   b) obtaining an X-ray crystal diffraction pattern of the soaked macromolecule crystal; and

c) using said X-ray crystal diffraction pattern to identify any compound bound to the macromolecule crystal, said compound being a ligand of the target macromolecule.

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In this method, the synthesis of the collection of compounds will take place in a single reaction vessel, i.e. the vessel in which the solution containing one or more crystals of the target macromolecule is present.

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'Just-in-time' synthesis involves synthesizing the collection of compounds remotely from the solution which contains the one or more crystals of the target protein, and then transferring the synthesized collection into the solution containing the one or

20   more crystals of the target protein. No purification and/or characterisation of the synthesized collection is carried out. The synthesis may take place in a solvent which is not compatible with the macromolecule crystals, from which the collection of compounds must be separated in order to add them to the solution

25   containing the macromolecule crystals.

Accordingly, a second aspect of the present invention provides a method for identifying a ligand of a target macromolecule comprising the steps of:

30   a) synthesizing a collection of unpurified compounds suitable for screening against a target macromolecule

b) adding the collection of compounds to a solution containing one or more crystals of the target macromolecule;

35   c) obtaining an X-ray crystal diffraction pattern of the soaked macromolecule crystal; and

d) using said X-ray crystal diffraction pattern to identify

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any compound bound to the macromolecule crystal, said
compound being a ligand of the target macromolecule.

In this method, the synthesis of the collection of unpurified
5   compounds may occur in one or more reaction vessels.

If step a) takes place in a solvent which is not compatible with
the macromolecule crystals then after step a) the collection of
compounds is separated from the solvent in which the compounds
10   were synthesised.

Typically, such non-compatible solvents are organic and the step
of separating the collection of compounds from this solvent is
usually carried out by evaporating the solvent. This is then
15   followed by re-dissolution of the collection of compounds in the
solution containing the one or more macromolecule crystals.

Enzyme catalysis in organic solvents has attracted much interest
in recent years (Mattos C. and Ringe D., *Curr Opin Struct*
20   *Biol.*,11(6), 761-4) and the use of enzymes in non-aqueous media
has extended the field of biocatalysis (*ASGSB Bull.*, 4(2),125-132
(1991)). Much work has been done to map out organic binding sites
in crystals by soaking the crystals in organic solvents (English,
A.C., *et al.*, *Proteins*, 37, 628-640 (1999); Mattos C. and Ringe
25   D., *Nauret Biotechnol.*, 14(5), 595-9 (1996)). In addition it has
been shown that enzyme crystals can retain activity in organic
solvents, both in the presence and absence of crosslinking agents
(Ayala, M., *et al.*, *Biochem. Biophys. Res. Comm.*, 295(4), 828-31
9 (2002)).
30

An alternative approach is to separate the non-compatible solvent
from the solution containing the one or more macromolecule
crystals by a permeable membrane, which allows transfer of the
compounds in the collection from the non-compatible solvent to
35   the solution containing the one or more macromolecule crystals.
This approach requires a membrane which is porous enough to allow
the diffusion of the synthesised compounds from the solvent in

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which they were synthesised to the solution containing the one or
more macromolecules, whilst substantially preventing any
diffusion of the solvents. Dialysis buttons provide one means by
which this can occur, and are available, for example, from
5   Hampton Research. Their use is described in 'Crystallisation of
Nucleic Acids and Proteins, edited by Ducruix, A. and Giege, R.,
The Practical Approach Series, Oxford University Press, 1992.

The ligands identified by the methods of the present invention
10   may be subsequently modified to alter their binding to the target
macromolecule or to improve their usefulness as a pharmaceutical.
Such modification is conventional in the art. Possible
modifications include: substitution or removal of groups
containing residues which interact with the target macromolecule,
15   for example groups which interact with the amino acid side chain
groups of a protein; the addition or removal of groups in order
to decrease or increase the charge of a group in a compound; the
replacement of a charge group with a group of the opposite
charge; or the replacement of a hydrophobic group with a
20   hydrophilic group or vice versa. Additionally, a group may be
replaced with another retaining similar properties but that
better occupies the cavity in the macromolecule increasing the
surface of the ligand in contact with the macromolecule cavity.
This may be achieved using the methodologies disclosed in this
25   invention, or by conventional synthetic approaches typically
utilised by those skilled in the art of medicinal chemistry.
Many of these changes will improve the usefulness of a compounds
as a pharmaceutical. It will be understood that these are only
examples of the type of substitutions considered by medicinal
30   chemists in the development of new pharmaceutical compounds and
other modifications may be made, depending upon the nature of the
starting compound and its activity.

Without wishing to be bound by theory, the detection of the
35   ligand bound to the target macromolecule relies on the occupancy
in the macromolecule crystals of one of the highest affinity
ligands, this being driven by ligand-macromolecule interactions.

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This method avoids disadvantages associated with biological
screening methods in which the alteration of macromolecule
5   activity by a potential ligand is assessed, as in that case
compounds which bind weakly but non-specifically can alter
macromolecule activity in a non-selective manner. Such non-
selective inhibition produces false-positives, as the assay shows
protein activity inhibition, but the compound would perform no
10   useful function as a drug, as it would interfere with the
activity of other proteins. Only compounds bound in a binding
site will be detected by the present method. In particular, only
compounds bound in a binding site with resolvable occupancy will
be detected by the present method.

15

Binding sites are sites within a macromolecule, or on its
surface, at which ligands can bind. Examples are the catalytic or
active site of an enzyme (the site on an enzyme at which the
amino acid residues involved in catalysing the enzymatic reaction
20   are located), allosteric binding sites (ligand binding sites
distinct from the catalytic site, but which can modulate
enzymatic activity upon ligand binding), cofactor binding sites
(sites involved in binding/co-ordinating cofactors e.g. metal
ions), or substrate binding sites (the ligand binding sites on a
25   protein at which the substrates for the enzymatic reaction bind).
There are also sites of protein-protein interaction. If the
macromolecule is a nucleic acid, then binding sites may be the
bases of the nucleic acid, or spaces in their structures, e.g.
the major or minor grooves in the helical DNA, interactions with
30   phosphate, ribose or deoxy ribose groups or intercalated between
the bases.

The present method also enables screening where the target
macromolecule has more than one active site, as the data for each
35   site can be analysed independently from the other sites to
determine the compound bound in that site. In such cases, the
information the method of the invention provides on the binding

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of two or more separate ligands to the target macromolecule can
be used in the linked-fragment approach to drug design, in a
similar manner to the method described by Greer, et al., J. Med.
Chem., 37(8), 1035-1054 (1994) for the synthesis of a thymidylate
5   synthase inhibitor series. The basic concept behind linked-
fragment approaches to drug design is to determine
(computationally or experimentally) the binding location of
plural ligands to a target molecule, and then to construct a
molecular scaffold to connect the ligands together in such a way
10   that their relative binding positions is preserved. The methods
of synthesis and screening of the present invention may then be
used to determine the best ligands from a library of such
compounds, or individual compounds binding ability can be
assessed using known methods.
15

Even if the present invention only provides information on the
binding of ligands at a single binding site of a target
macromolecule, a structure-based approach can be used to develop
ligands which interact with further binding sites. Such a
20   fragment growing approach is described in Blundell, T., et al.,
Nature Reviews Drug Discovery, vol. 11, 45-54 (2002).

It is preferred that the members of the collection of compounds
are present at a concentration of at least 5 to 50 times,
25   typically at least 10, their K_i (depending case by case with the
macromolecule used) so that the occupation of the binding site in
the target macromolecule will not depend on the relative
quantities of each compound in the collection.

30   In the case of competitive binding of a ligand to a macromolecule
K_i is defined as:

$$K_i = \frac{[M][L]}{[ML]}$$

where [M] is the concentration of the macromolecule, [L] is the
concentration of free ligand, [ML] is the concentration of the
35   ligand-macromolecule complex.

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Where inhibitors are binding in an uncompetitive or non-competitive fashion the K_i is defined as in Fundamentals of Enzyme Kinetics by A. Cornish-Bowden, Portland Press, 1995, ISBN 1 85579 0720, which is herein incorporated by reference.

5

It is preferred that the amount of each compound, being a member of the collection of compounds, present in the solution will be present at a concentration which is at least 5 or 10 times as much as the concentration of the target macromolecule in the

10   reaction system, more preferably 100, 1 000 or even 10 000 times the concentration of the target macromolecule in the reaction system.

The binding of the ligands to the target macromolecule may be

15   through non-covalent interactions or covalent bonding. If the target macromolecule is a protein, then covalent binding of the ligand to the protein may occur when the active site of the protein contains a catalytic residue such as in serine and cysteine proteases. If the target macromolecule is a nucleic

20   acid, then certain classes of compounds are known to interact by covalent binding, e.g. pyrrolobenzodiazepines covalently bind to the exocyclic amino group of guanine.

In some embodiments of the present invention, it is preferred

25   that the members of the collection of compounds do not bind covalently to the target macromolecule, but that they interact through non-covalent binding.

Further aspects of the invention relate to any novel compounds

30   disclosed herein, their use as pharmaceuticals and their use in methods of therapy. In particular, further aspects of the invention include:
    a)   a ligand identified by the method of the present invention, or salts, solvates and chemically protected forms thereof;

35   b)   a pharmaceutical composition comprising a ligand identified by the method of the present invention, or salts, solvates and chemically protected forms thereof, and a pharmaceutically

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acceptable carrier or diluent;

c)   the use of a ligand identified by the method of the present invention, or salts, solvates and protected forms thereof, in a method of treatment of the human or animal body;

d)   the use of a ligand identified by the method of the present invention, or salts, solvates and protected forms thereof, in the manufacture of a medicament for the treatment of a disease ameliorated by the binding of a ligand to the target macromolecule used in the method of the invention; and

e)   a method for the treatment of a disease ameliorated by the binding of a ligand to the target macromolecule used in the method of the invention comprising administering to a subject suffering from said disease a therapeutically-effective amount of a ligand identified by the method of the present invention, or salts and solvates.

Suitable carriers and diluents and information on pharmaceutical compositions can be found in standard pharmaceutical texts, for example, Handbook of Pharmaceutical Additives, 2nd Edition (eds. M. Ash and I. Ash), 2001 (Synapse Information Resources, Inc., Endicott, New York, USA); Remington's Pharmaceutical Sciences, 20th Edition, pub. Lippincott, Williams & Wilkins, 2000; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Further details of the invention will now be presented by way of explanation and example.

Although the discussion below focuses on the purification, crystal growth, X-ray crystallography and determination of ligand structure when the macromolecule is a protein, the techniques described are, in general, applicable to other macromolecules, such as nucleic acids and complexes, with appropriate modifications as known to the person skilled in the art.

Target Protein Purification.

A specific target protein can be isolated from animal, plant, or bacterial sources directly, or via recombinant methods.   The

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generation of recombinant protein, using systems such as insect
cells (e.g. *S. frugiperda*, or *Drosophila* cells), *E. coli*, yeast
(*S. cerevisiae, S. pombe, P. Pastoris,* etc) or modified human
cell lines, means that truncated, or otherwise genetically

5   engineered, proteins can be generated. A protein crystallography
project to obtain crystals normally necessitates access to a
recombinant protein production system, but the method of the
present invention may be performed with a single crystal, which
may constitute, for example, between 0.1 and 100 μg.

10

It is generally accepted that the higher the degree of purity and
homogeneity of a protein preparation the easier that it will be
to grow protein crystals from the preparation. Protein purity
reflects the number of protein species within a preparation. It

15   also refers to the number, and nature, of any other non-protein
species present (e.g. low molecular weight contaminants). An
ideal protein preparation should contain solely one protein
species, or one species of protein complex, in which all the
protein molecules, or protein complexes, are identical in terms

20   of their amino acid composition, mass etc. The purity of a
protein preparation may be gauged via a variety of experimental
techniques such as sodium dodecyl sulphate page (SDS-page) gels,
mass spectrometry, antibody binding and detection (Western
blotting), etc.. Protein purities in excess of 90% are often

25   deemed acceptable for crystallisation trials, but practitioners
of the art of protein purification will generally try and strive
for purities in excess of this arbitrary threshold, due to the
perceived benefits of maximising protein purities.

30   Within a protein preparation, homogeneity can refer to the degree
of uniformity observed for parameters such as the stoichiometry
of proteins in a multiprotein complex, the mono-dispersity of the
protein/complexes in solution, the oxidation, or protonation,
state of amino-acid side chains, within proteins, the uniformity

35   of post translational modifications (e.g. are all protein
molecules within the population equivalently phosphorolated,
glycosylated, or have any essential co-factors been uniformly and

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correctly incorporated) and the protein conformations that exist
within a given population of protein molecules/complexes. The
homogeneity of a protein preparation may be probed using a
multitude of experimental methods some of which are: mass-
5   spectrometry, Western blotting, SDS-page, analytical
ultracentrifugation, size-exclusion chromatography, affinity
chromatography, ion-exchange chromatography, hydrophobic
interaction chromatography, surface plasmon resonance, activity
assay, electron microscopy, dynamic light scattering (DLS), N-
10   terminal sequencing, iso-electric focussing (IEF), proteolytic
digest, fluorescence, circular dichroism (CD), native gel
electrophoresis, bandshift assays, or nuclear magnetic resonance
(NMR). Maximising the degree of homogeneity within a protein
preparation is again deemed desirable, as maximising homogeneity
15   is also believed to positively correlate with maximising
crystallisability.

The Growth of Protein Crystals
Crystallisation of any species requires the formation of a
20   supersaturated solution of the species in question and a
nucleation event that is capable of initiating crystal growth.
Post-nucleation the ambient conditions must be such that crystal
growth can be sustained until the physical dimensions and
properties of the crystals thus obtained are adequate for any
25   subsequent experimental procedures required. Protein molecules
typically only retain their structural integrity within an
aqueous environment. Therefore protein crystals are normally
grown in the aqueous phase. Protein crystals may grow if a
nucleation event occurs in a pure and homogeneous protein
30   solution that has been driven to a state of super-saturation.

Protein crystallisation is generally attempted using the vapour
diffusion (sitting drop, hanging drop, sandwich drop, pH gradient
etc), dialysis, batch, micro-batch, liquid-liquid diffusion, or
35   in-gel-crystallisation methods. All these methodologies have
been extensively described (Protein Crystallisation: Techniques,
Strategies and Tips, Edited by T.M. Bergfors, IUL Biotechnology

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Series, 1999, Published by International University Line, La
Jolla, California, ISBN: 0-9636817-5-3). All of the afore
mentioned crystallisation processes function by generating a
supersaturated protein solution, which promotes the spontaneous
5 formation of crystallisation nuclei, and which is then
subsequently able to sustain crystal growth.

There are diverse physico-chemical parameters that can influence
whether or not a protein construct, or protein complex, will
10 crystallise. Typically each protein crystallizes under a unique
set of conditions, which cannot be predicted in advance. Simply
driving the protein concentration to super-saturation, to bring
it out of solution, will generally not work. The result would,
in most cases, be an amorphous precipitate. Some parameters that
15 may be varied are: the pH of solutions, the choice and
concentration of buffer (if any) (e.g. Phosphate, MES, BIS-TRIS,
TRIS, BES, PIPES, HEPES, MOPS, BICINE, CHES, CAPS etc),
temperature, choice of crystallisation method (see above), volume
of crystallisation, protein concentration, the addition of
20 reducing agents (e.g. DTT, β-mercaptoethanol), detergents (e.g.
decyl-β-D-maltoside, dodecyl-β-D-maltoside, ocytl-β-D-
glucopyranoside, decanoyl-N-methylglucamide, Triton,
octyltetraoxyethylene ether, etc.), alcohols (e.g. ethanol,
isopropanol, methanol, 2-methyl-2,4-pentanediol (MPD)), salts
25 (e.g. chlorides, acetates, sulphates, phosphates, bromides,
iodides, fluorides, nitrates, bicarbonates, chlorates, chromates,
citrates, tartrates, cacodylates, formates, hydroxides, etc.),
polyethylene glycols (PEGS), ethylene glycols, methoxy
polyethylene glycols (MPEGS), heavy atoms and ions (e.g. iron,
30 copper, zinc, cobalt, manganese, nickel, tungstates, vanadates,
sodium, magnesium, potassium, lithium, calcium, aluminium, Xenon,
etc.), or other additives such as dimethylsulfoxide (DMSO),
denaturants (e.g. urea, guanadinium chloride, etc.), glycerol,
sulfabetaines, jeffamines, AMPPNP, ATP, ADP, GTP, GDP peptides,
35 tertiary-butanol, amino acids, azides, DNAs, RNAs, sugars,
lipids, drugs, etc..

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There are numerous crystallisation kits available (e.g. from Hampton Research), which attempt to broadly sample as many parameters in crystallization space as possible. In many cases these general screens help to identify a starting point for
5   crystallisations in the form of crystalline precipitates and/or rough, or micro-, crystals. Typically these crystals are unsuitable for direct diffraction analysis and require further optimisation. Successful crystallization can be aided by knowledge of a protein's behaviour in terms of solubility,
10   dependence on metal ions for correct folding or activity, interactions with other molecules and any other data that are available.

Systematically screening such a large number of parameters
15   represents an extremely complex multi-dimensional search problem and is, as such, exceptionally difficult to perform in a systematic manner. Even with the advent of automated protein crystallisation it is often the case that crystallisation of a protein will require a very high degree of human input and the
20   impact of intangible parameters such as serendipity, insight, and random error.

If preliminary crystals have been obtained it is often necessary to further modify the crystallisation conditions in an attempt to
25   simultaneously maximise the internal order and physical ·dimensions of the crystals grown. Optimising these parameters is deemed beneficial for helping to maximise the data quality obtained in subsequent X-ray diffraction experiments. Identification of a set of initial crystallisation conditions
30   reduces the potential parameter space that has to be explored, but crystal optimisation can still remain a time consuming and laborious process. Techniques such as macro- or micro-seeding may also aid crystal optimisation.

35   Details of some of the proteins crystallised, and information on some of the protein crystallisation conditions identified, are contained, for example, within the following internet databases:

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http://wwwbmcd.nist.gov:8080/bmcd/bmcd.html (Gilland, G.L., *et al., Acta Crystallogr.*, D50, 408-413 (1994));

http://xray.bmc.uu.se/embo/structdb/links.html;

http://www.mpibp-

5   frankfurt.mpg.de/michel/public/memprotstruct.html;

http://www.rcsb.org/pdb/ (Berman, H.M., *et al., Nucleic Acids Research*, 28, 235-242 (2000));

http://www.ebi.ac.uk/msd/

and have been described in the following publications:

10   Blundell, T., *et al.*, "Protein Crystallography", Academic Press, New York (1976); McPherson, *et al.*, *"Preparation and Analysis of Protein Crystals"* in "Preparation and Analysis of ProteinCrystals", John Wiley & Sons, New York (1982); Carter, *et al.*, *"Design of crystallization experiments and protocols."*,

15   pages 47-71 in "Crystallization of Nucleic Acids and Proteins - A Practical Approach", (Ducruix, A. & Giege, R., eds) IRL Press, Oxford (1992); Ducruix, A., *et al.*, *"Methods of crystallization."*, pages 73-98 in "Crystallization of Nucleic Acids and Proteins - A Practical Approach", (Ducruix, A. & Giege,

20   R., eds) IRL Press, Oxford (1992); "Protein Crystallisation: techniques, stratagies, and tips." IUL Biotechnology Series (1999), ISBN 0-9636817-5-3.

Obtaining X-ray diffraction data from soaked protein crystals

25   An X-ray diffraction experiment consists of exposing a protein crystal to a collimated, coherent, beam of X-rays and recording the resulting X-ray diffraction pattern. A diffraction pattern arises from the elastic scatter of X-rays off electrons within the planes of atoms within a protein crystal. The mathematics

30   underlying X-ray diffraction may be represented in their simplest form by the Bragg equation:

$$n\lambda = 2d\sin\theta$$

where n is an integer, λ is the wavelength of the incident X-rays; θ is the scattering angle of the X-rays off a given plane

35   of atoms, and d is the Bragg spacing, or spacing between successive planes of atoms (Bragg planes). Thus as long as λ corresponds to atomic scales (i.e. $\approx 1\text{Å}$) then atomic scale

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features should be discernable within an electron density map
calculated using the diffraction data. Typically it is considered
that X-ray data are required to a Bragg spacing of ≤3 Å, for a
given X-ray wavelength, if the data from an X-ray diffraction
5   experiment are to be of use in determining the atomic positions
within a structure. During an experiment the various Bragg planes
within a crystal will only satisfy the mathematical criteria
necessary for diffraction at specific crystal orientations
relative to the incident X-ray beam.   So as to obtain
10   diffraction data relating to as many Bragg planes as possible the
crystal is rotated in the X-ray beam. Thus all possible plane
orientations are explored.   The angle through which a crystal
must be rotated in order to obtain a complete set of data
relating to a specific Bragg spacing is defined by the space
15   group of the crystal and also the initial orientation of the
crystal in the X-ray beam.   The smallest Bragg spacing for which
diffraction data are available is termed the resolution of the
experiment.   It is desirable to maximise the data completeness
for an experiment.   That is, data should ideally be 100% complete
20   up to the resolution limit of the experiment.

Unfortunately the high energy of X-ray photons means that they
cause damage to protein molecules. This is thought to be at
least partially due to the generation of ions and free radicals
25   within the crystals. Prolonged exposure of a protein containing
crystal to an X-ray beam will thus result in a deterioration and
decay of the proteins. This is typically manifested by a decline
in the data resolution and quality. This problem may be
partially circumvented by cryogenically freezing protein
30   containing crystals in vitreous ice at 100K. Freezing of the
crystal means that any ion, or free radical, species that are
generated are unable to migrate through the crystal. Thus the
longevity of the crystal in the X-ray beam is extended and the
data quality and resolution typically improved relative to an
35   unfrozen data collection. Normally protein containing crystals
cannot be directly frozen in the solutions in which they grew
(mother liquor). This is because direct freezing often leads to

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the formation of ice crystals within the mother liquor. These
ice crystals can destroy the internal order within a protein
crystal and thus abolish diffraction. The addition of a cryo-
protectant to the protein's mother liquor can, however, lead to
5   the formation of vitreous ice on freezing. This should not
destroy the internal order of a protein crystal and thus retain
diffraction from the crystal. Normally protein containing
crystals must be transferred from their mother liquor into a
specially formulated cryo-protectant solution prior to freezing.
10   The exact composition of the cryo-protectant solution, the
transfer protocol, and the freezing protocol must be uniquely
determined for each crystal system and often for each experiment.

Determination of ligand structure

15   A mathematical operation termed a Fourier transform relates the
diffraction pattern observed from a crystal and the molecular
structure of the protein and ligand comprising the crystal
(Blundell, T., et al., "Protein Crystallography", Academic Press,
New York (1976); Drenth, "Principles of Protein X-ray
20   Crystallography", Springer (1994)). A Fourier transform may be
considered to be a summation of sine and cosine waves each with a
defined amplitude and phase. Thus, in theory, it is possible to
calculate the electron density associated with a protein and
ligand structure by carrying out an inverse Fourier transform on
25   the diffraction data. This requires amplitude and phase
information to be extracted from the diffraction data. Amplitude
information may be obtained by analysing the intensities of the
spots within a diffraction pattern. The conventional methods for
recording diffraction data do, however, mean that any "phase
30   information" is lost. This phase information must be in some way
recovered and the loss of this information represents the
"crystallographic phase problem". The phase information
necessary for carrying out the inverse Fourier transform can be
obtained via a variety of methods.
35

If the structure of the unsoaked protein is already available, as
would normally be the case, a set of theoretical amplitudes and

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phases may be calculated using the protein model and then the theoretical phases combined with the experimentally derived amplitudes. An electron density map may then be calculated and the protein and ligand structure observed. Electron density maps
5   can be calculated using programs such as those contained in the CCP4 computing suite (Collaborative Computational Project 4, *Acta Crystallographica*, D50, 760-763 (1994)). For map visualization and model building programs such as "O" (Jones, *et al.*, *Acta Crystallographica*, A47, 110-119 (1991)) or "QUANTA" (Jones, *et*
10 *al.*, (1991) and commercially available from Accelrys, San Diego, California) can be used.

An alternative approach employs (i) X-ray crystallographic diffraction data from the complex of ligand and protein and (ii)
15 a three-dimensional structure of the unsoaked protein, to generate a difference Fourier electron density map of the complex. The difference Fourier electron density map may then be analysed to identify the ligand.

20 Analysis of electron density maps may be aided by software, for example, AutoSolve® (Blundell, T., *et al.*, *Nature Reviews Drug Discovery*, 11, 45-54 (2002)) or the ligand fitting module in QUANTA, XLIGAND (QUANTA: see above; X-LIGAND: Oldfield, T.J., *Acta Crystallogr D Biol Crystallogr.*, 57(5), 696-705 (2001)).
25

If there is no known structure of the protein then alternative methods for obtaining phases must be explored so as the resolve the structure of the unsoaked protein (Blundell, T., *et al.*, "Protein Crystallography", Academic Press, New York (1976)). One
30 method is multiple isomorphous replacement (MIR). This relies on soaking "heavy atom" (i.e. Platinum, Uranium, Mercury, etc) compounds into the crystals and observing how their incorporation into the crystals modifies the spot intensities observed in the diffraction pattern. An alternative method for obtaining phase
35 information for a protein of unknown structure is to perform a multi-wavelength anomalous dispersion (MAD) experiment. This relies on the absorption of X-rays by electrons at certain

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characteristic X-ray wavelengths. Anomalous scattering by atoms
within a protein will modify the diffraction pattern obtained
from the protein crystal. Thus if a protein contains atoms which
are capable of anomalous scattering a diffraction dataset
5   (anomalous dataset) may be collected at an X-ray wavelength at
which this anomalous scattering is maximal. The most usual way
to introduce anomalous scatterers into a protein is to replace
the sulphur containing methionine amino acid residues with
selenium containing seleno-methionine residues. This is done by
10   generating recombinant protein that is isolated from cells grown
in controlled growth media that contains seleno-methionine
(Doublie, S., *Methods in Enzymology*, 276, 523-530 (1997)).
Selenium is capable of anomalously scattering X-rays and may thus
be used for a MAD experiment. Another method generally available
15   for the calculation of the phases necessary for the determination
of an unknown protein structure is molecular replacement. This
method relies upon the assumption that proteins with similar
amino acid sequences (primary sequences) will have a similar fold
and three-dimensional structure (tertiary structure). Examples
20   of computer programs known in the art for performing molecular
replacement are CNX (Brunger, A.T., *et al.*, *Current Opinion in
Structural Biology*, 8(5), 606-611 (1998) and also commercially
available from Accelerys San Diego, CA) or AMORE (Navaza, J.,
Acta Cryst., **A50**, 157-163 (1994)). The phase information
25   obtained by one of these means, when combined with the
experimentally obtained amplitudes from the native dataset,
enables an electron density map of the unknown protein molecule
to be calculated using the Fourier transform method.

30   If an electron density map has been calculated for a protein of
unknown structure then the amino acids comprising the protein
must be fitted into the electron density for the protein. This
is normally done manually, although high resolution data may
enable automatic model building. The process of model building
35   and fitting the amino acids to the electron density can be both a
time consuming and laborious process. Once the amino acids have
been fitted to the electron density it is necessary to refine the

- 20 -

structure. Refinement attempts to maximise the correlation
between the experimentally calculated electron density and the
electron density calculated from the protein model built
(Blundell, T., et al., "Protein Crystallography", Academic Press,
5   New York (1976) and "Methods in Enzymology", vols. 114 & 115,
Wyckoff, H.W., et al., eds., Academic Press (1985)). Refinement
also attempts to optimise the geometry and disposition of the
atoms and amino acids within the user-constructed model of the
protein structure. Sometimes manual re-building of the structure
10   will be required to release the structure from local energetic
minima. There are now several software packages available that
enable an experimentalist to carry out refinement of a protein
structure such as CNX (see above), or REFMAC (Murshudov, G.N., et
al., Acta Crystallographica, D53, 240-255 (1997)). There are
15   certain geometry and correlation diagnostics that are used to
monitor the progress of a refinement. These diagnostic
parameters are monitored and rebuilding/refinement continued
until the experimenter is satisfied that the structure has been
adequately refined.

20

The atomic coordinate data of the co-complexes formed from the
methods of the invention can be routinely accessed using computer
programs, for example, RASMOL (Sayle, et al., TIBS, 20, 374
(1995)), which is a publicly available computer software package,
25   which allows access and analysis of atomic coordinate data for
structure determination and/or rational drug design or
AstexViewer™ which is contained in the CCP4 computing suite.

Information from X-ray crystallography

30   The information obtained by the method according to the invention
can be used to provide much more information that the identity of
the ligand which has bound. As the information on the ligand
results from fitting to the electron density measured in the
protein active site, this can allow the mode of binding and
35   interactions to be ascertained, which can be useful in further
elaboration and optimisation of the ligand.

The collections of compounds

The collection of compounds for use in the present invention are
produced by one or more synthetic processes designed to connect
two or more sets of monomers together. Monomers are molecules
5   which share a common reactivity that makes them capable of
combining with another complementary monomer to form a larger
compound. Each set of monomers will contain at least one monomer
and preferably no more than 100 monomers, such that between about
5 and about 1000 compounds are in each collection of compounds.
10   It is preferred that all the compounds in a collection comprise a
common functional group (sometimes referred to as a 'templating
moiety') which is produced by the reaction of two or more
complimentary functional groups present on the monomer sets used
in the synthetic process. In one preferred embodiment each
15   compound in a collection is related to other members of the
collection by virtue of being synthesised from at least one
common monomer unit. By having common features or trends in the
structures of the compounds in the collection, it makes it
possible to identify which moieties in the compounds (derived
20   from individual monomer units) bind best to the target protein,
even if the independent monomer itself does not have any
detectable binding. This is achieved by observing the preference
for binding that the macromolecule exhibits for compounds from
the collection.
25

It is further preferred that the collection of compounds will
have shape features that differ sufficiently to allow at least
one set of monomers used in their production to be distinguished
from each other. This then allows determination of the chemical
30   structure of the bound ligand, or to at least determine part of
its structure. If only part of its structure can be determined,
re-synthesis of some of the members of the collection of compound
that contain this partial structure will be necessary and these
compounds are soaked into the crystal as singlet experiments so
35   that the chemical structure of the bound ligand or ligands can be
determined. This type of re-synthesis approach is termed a
chemical deconvolution and is well known to practitioners of

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combinatorial or parallel synthesis to identify the biologically
active member from a mixture of synthesised compounds.

'In-situ' synthesis

5   As mentioned above, protein crystals are sensitive to the media
     in which they are grown and kept, and therefore the methods
     chosen for 'in-situ' synthesis, i.e. synthesis of the collection
     of compounds in the presence of the target protein crystals, have
     to be chosen such that they can occur under conditions which will
10   not destroy the target protein crystal. If some degradation of
     the crystals occur, this should not be to such an extent that the
     X-ray diffraction results are not of sufficient quality to allow
     for identification of the ligand (see above).

15   Typically reactions are those which can be carried out in a
     purely aqueous media at around room temperature (4 to 30°C), and
     at a pH of 4 to 10.

     Examples of these chemistries, which apply to both methods of
20   combinatorial synthesis and parallel synthesis, include:
     Acetal or Ketal formation; Addition reactions; Aldol
     condensations and related condensation reactions; Allylations;
     Cycloaddition reactions; Disulfide formation; Hydrazone
     formation; Mannich reactions; Michael reactions and related
25   Conjugate Addition reactions; Palladium mediated reactions;
     Reductive alkylation; Substitution reactions; and Three or Four
     Component Reactions.

     The following reactions can be carried out using synthetic
30   procedures described in general organic chemistry texts such as
     March's Advanced Organic Chemistry (Smith, M.B. and March, J., 5th
     edition, Wiley-Interscience, New York, 2001) or references given
     therein. Some synthetic reactions carried out in aqueous
     conditions have recently been reviewed by Ulf Lindstroem, see
35   "Stereoselective Organic Reactions in Water", *Chemical Reviews*,
     102(8), 2751-2771 (2002). The practice of combinatorial chemistry
   is described in references cited in publications by Roland Dolle

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(*Journal of Combinatorial Chemistry*, 4(5), 369-418 (2002);
Journal of Combinatorial Chemistry, 3(6), 477-517 (2001); *Journal
of Combinatorial Chemistry*, 2(5), 383-433 (2000); *Molecular
Diversity*, 4(4), 233-256 (2000); *Journal of Combinatorial
Chemistry*, 1(4), 235-282 (1999); *Molecular Diversity*, 3(4), 199-
233 (1998)).

These reactions take place between appropriate sets of ligand
precursor molecules ('monomers'), which are represented
schematically below, with M representing a substituent group that
is varied in each monomer set (e.g. M_1, M_2, M_3, etc.). Each
reacting set of monomers can have as few as a single member, up
to, for example, 40 members, although a maximum of about 20
members would be more usual. In some embodiments of the invention
it is preferred that each set of reacting monomers comprises at
least two monomers. A typical size for the resulting collection
of compounds would be between 5 and 1000, preferably between 5
and 100, with a range 10 or 20 to 50 or 70 being preferred.

In the following examples, R represents a substituent or H on a
monomer.

Acetal or Ketal formation
Addition of alcohols or a diol to an aldehyde or ketone,
catalysed by acid. The reactions are fully reversible leading to
thermodynamic products.
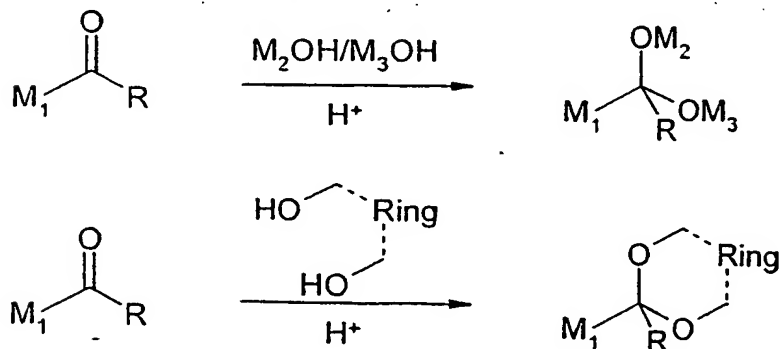
Addition reactions
For example, addition to an epoxide under acidic conditions, or

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addition of alcohols to enol ethers.
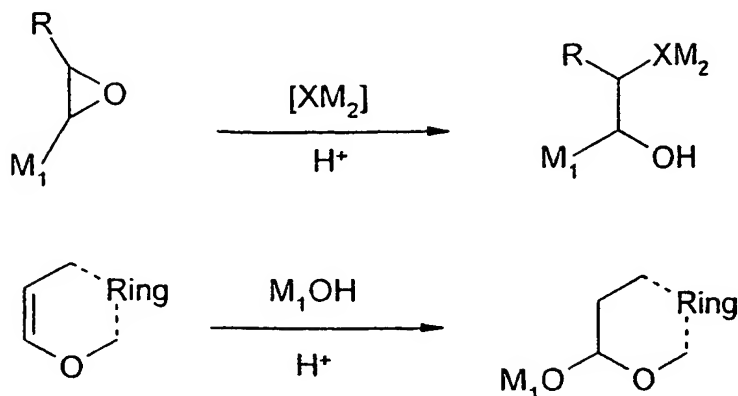
X = O, S, N or P, and where the ring may be optionally
substituted on any available position.

5

Aldol condensations and related condensation reactions
For examples, see Kobayashi, S. and Manabe, K., *Accounts of
Chemical Research*, **35(4)**, 209-217 (2002).
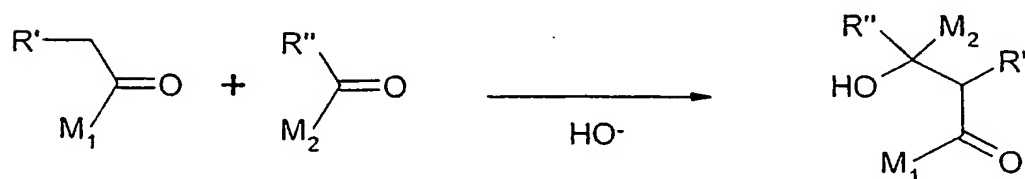
10

Related condensation reactions are the Knoevenagel reaction, the
Peterson reaction, the Perkin reaction, the Darzen's reaction,
Tollens' reaction, the Wittig reaction and the Thorpe reaction.
Careful selection of the monomers is required in order for these
15   reactions to proceed under aqueous conditions.

Allylations
For examples, see Kobayashi, S. and Hachiya, I., *Yuki Gosei
Kagaku Kyokaishi*, **53(5)**, 370-80 (1995).

20

Cycloaddition reactions
For example, the Diels-Alder reaction, see Fringuelli, F., et
al., *European Journal of Organic Chemistry*, **3**, 439-455 (2001).
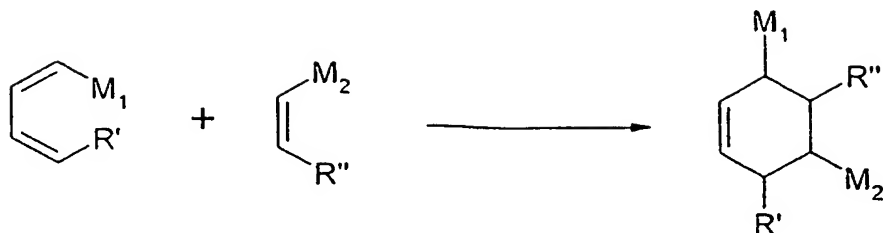
Many variations exist of the above formula, including where
heteroatoms are incorporated (e.g. aza-Diels-Alder reactions).
Cycloadditions to form 5 membered ring systems are also very

5   general and an illustrative example is the cycloaddition of
nitrile oxides with alkynes to form oxazoles which occurs at room
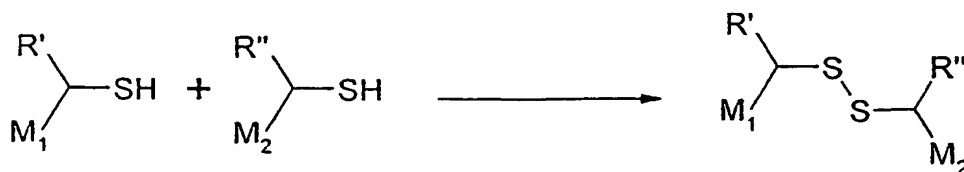temperature under very mild conditions.

10   An example of a cycloaddition reaction that is water-tolerant is
the "click chemistry" described by Sharpless in Lewis, *et al.,*
Angew. Chem. Int. Ed., **41(6)**, 1053-1057 (2002). In this an azide
and an acetylene undergo a Huisgen 1,3-dipolar cycloaddition to
give 1,2,3-triazoles.

15
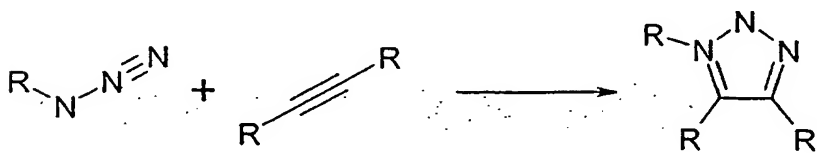
Disulfide formation
Occurs reversibly under very mild conditions.

20

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Hydrazone formation

Occurs reversibly under very mild conditions.
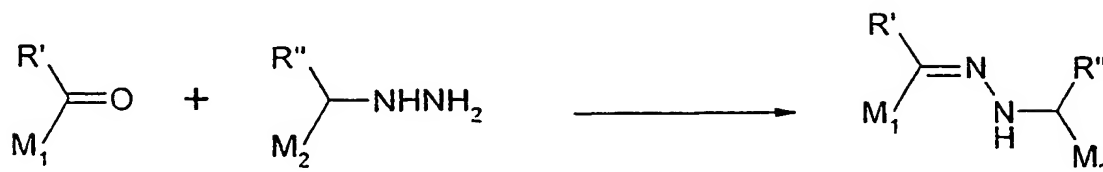
5   *Mannich reactions*

For examples, see Akiyama, T., *et al.*, *Advanced Synthesis &*
Catalysis, **344(3+4)**, 338-347 (2002).
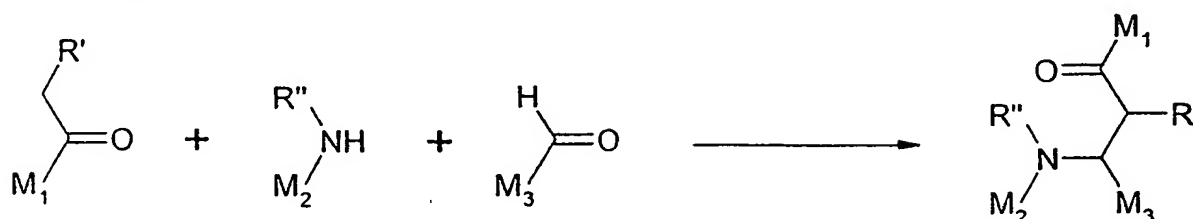
10   *Michael reactions and related Conjugate Addition reactions*
Addition of nucleophiles to α,β-unsaturated carbonyl compounds is
another example of addition reactions suitable for use in the
invention.
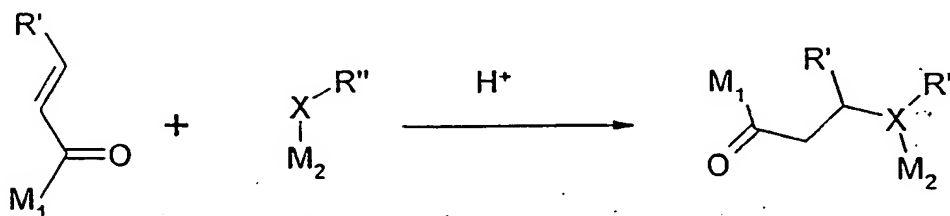
15   The carbonyl can also be replaced by other electron withdrawing
groups, such as nitro groups, see Da Silva, F. and Jones, J.,
Journal of the Brazilian Chemical Society, **12(2)**, 135-137 (2001).

A related transformation is the Baylis Hillman reaction, see Yu,
20   C., *et al.*, *Journal of Organic Chemistry*, **66(16)**, 5413-5418
(2001).

Palladium mediated reactions
Many palladium mediated reactions can be carried out in aqueous
25   media, e.g. Heck, Sonogashira, Tsuji-Trost, Suzuki, Stille, see

Pierre Genet, J. and Savignac, M., *Journal of Organometallic Chemistry*, 576(1-2), 305-317 (1999). A representative illustration is the Suzuki cross coupling reaction:
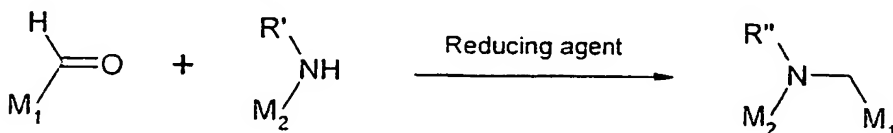
$$M_1(Aryl)\!\!-\!\!BOH_2 \; + \; M_2(Aryl)\!\!-\!\!Hal \xrightarrow{\;\;Pd(0)\;\;} M_1\!\!-\!\!M_2$$
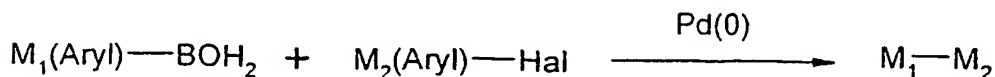
5

Reductive alkylation

Occurs under very mild conditions. An example carried out in the presence of a protein is Hochguertel, M., *et al.*, *Proceedings of the National Academy of Sciences of the United States of America*,

10   99(6),   3382-3387 (2002).

Substitution reactions

Many useful substitution reactions occur under aqueous

15   conditions, e.g. nucleophilic displacement (with alcohols, amines, thiols, carboxylic acids, enolates, hydrazines, dithianes etc) of alkyl halides, tosylates, mesylates and azides; ester, amide and urea formation by displacement of an activated ester or carbonate or carbamate; and aromatic nucleophilic substitution of

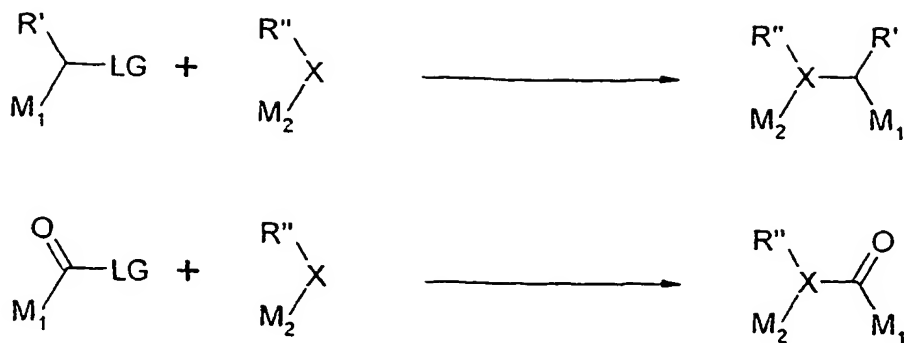20   electron deficient aromatic compounds with amines, alcohols, thiols etc.

An example of alkylation chemistry in the presence of a protein is Nguyen, R. and Huc, I., *Angew. Chem. Int. Ed.*, 40(9), 1774-6

25   (2001)

A generic scheme is as follows:

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Where LG is a leaving group, and X is a nucleophilic heteroatom
or carbon anion.

Three or Four Component Reactions
A number of multicomponent reactions proceed under mild mixed
aqueous conditions and are suitable for combinatorial library
design for the purposes of this invention. One example is the Ugi
condensation (see Domling, A., *Current Opinion in Chemical
Biology*, **6**(3), 306-313 (2002);
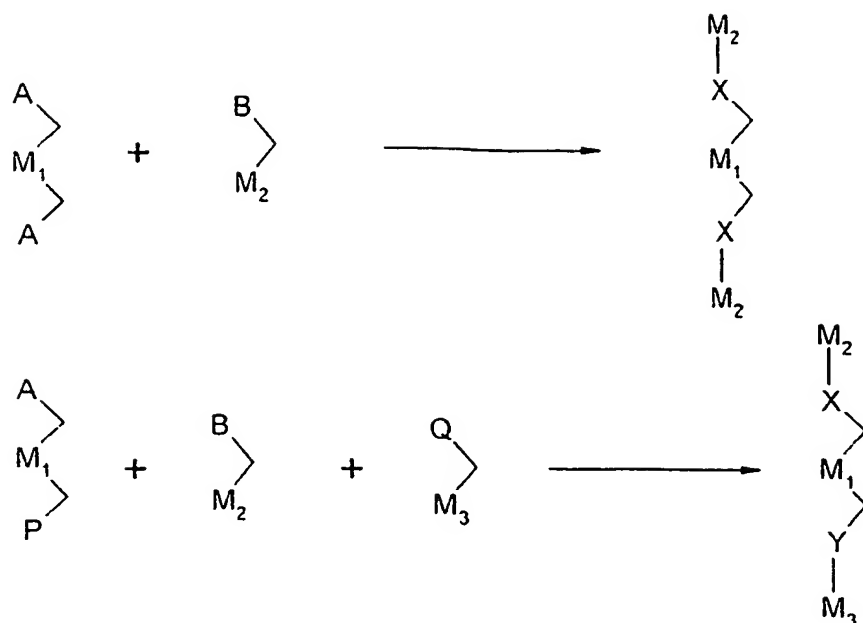Ugi, I., et al., *Combinatorial Chemistry*, 125-165 (1999)):
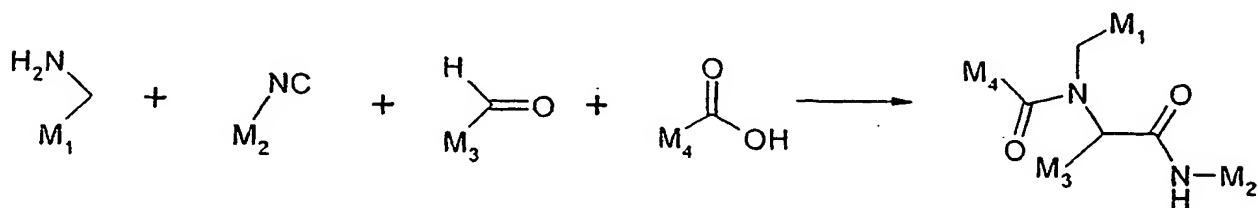
Also encompassed in the scope of the invention is design of
combinatorial reactions in which more than one functional group
can be present on any given monomer so that multimeric ligands
can be assembled. In this way two or more monomers can be
assembled by two or more functional group interconversions using
chemistry illustrated above or other chemistry possible under
mild aqueous conditions. For example, schematically:

Scheme 1:

$$A{-}M_1{-}A \;+\; B{-}M_2 \;\longrightarrow\; M_2{-}X{-}M_1{-}X{-}M_2$$

Scheme 2:

$$A{-}M_1{-}P \;+\; B{-}M_2 \;+\; Q{-}M_3 \;\longrightarrow\; M_2{-}X{-}M_1{-}Y{-}M_3$$

in which the monomer sets containing groups M_1 and M_2 react together through one chemistry (A+B=X) to give trimeric products containing the groups M_1 and M_2, and monomer sets containing M_1, M_2

5   and M_3 react together through two chemistries (A+B=X and P+Q=Y) to give trimeric products containing the groups M_1 and M_2 and M_3.

Other factors which need to be considered in designing suitable combinatorial library conditions under aqueous conditions are as

10   follows:

Solubility in water of the reacting monomers may be limiting to the efficiency of the transformations

Catalysis - Bronsted and Lewis acid catalysis and other catalysts may be used to allow a transformation to proceed in an

15   aqueous environment [For example Lindstroem, U., *Chemical Reviews*, 102(8), 2751-2771 (2002).]

Micelles - the use of micellar catalysts to enable the use of water as a solvent [For examples see Lindstroem, U. (2002)]

Cosolvents - the use of cosolvents to enable the use of water as

20   a solvent including, but not limited to, DMSO, polyethylene glycols, ethylene glycols, methanol, ethanol, isopropanol, acetone and acetonitile. These cosolvents should be compatible with the protein crystal and may typically be used in an amount of up to 20% of the solvent system, which is preferred, although

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an amount of up to 40% or higher may be possible.

Solubilisers - the use of solubilising agents to enable the use of water as a solvent in reactions of organic compoundds. [For examples see Lindstroem, U., (2002)]

5   *Surfactants* - the use of surfactants to enable the use of water as a solvent in reactions of organic compounds. [For examples see Lindstroem, U., (2002)] These surfactants should be compatible with the protein crystal.

10   The above described combinatorial library synthesis and procedures can similarly be adapted for mixed aqueous solvent conditions.

In some embodiments of the present invention, it is preferred
15   that the monomers cannot substantially bind to the target protein, but that only the collection of compounds formed include compounds that show affinity for the target protein. Preferably the ratio of the K_i of the strongest binding ligand formed to the K_i of the strongest binding monomer is at least 10 to 1, more
20   preferably 100, 1000 or even 10000 to 1.

In a preferred case, it is possible that the presence of the protein crystal in the solution in which the combinatorial chemistry takes place will exert an influence on the reactions
25   occurring. If the reactions are reversible, then without wishing to be bound by theory, this would allow generation of thermodynamic products having the advantage of allowing for the 'local enrichment' of products at the protein surface leading to formation of the most potent ligand possible in the library. The
30   principles of this effect are described by Huc, I. and Nguyen, R., *Combinatorial Chemistry and High Throughput Screening*, 109-130 (2001). If the reactions are irreversible, then without wishing to be bound by theory, this would allow generation of kinetic products having the advantage of 'local templating' of
35   products at the protein surface leading to formation of the most potent ligand possible in the library. The principles of this effect are described by Nguyen, R. and Huc, I., *Angew. Chem. Int.*

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Ed., 40(9), 1774-6 (2001) and in 'click chemistry' e.g. Lewis, W., et al., *Angew. Chem. Int. Ed.*, 41(6), 1053-1057 (2002).

'Just-in-time' synthesis

5　As 'just-in-time' synthesis is not carried out in the presence of the protein crystals, there is less restriction on the types of chemistries that can be used to generate the collections of compounds for screening.　However, in selecting appropriate starting materials and reaction conditions, it is preferred that
10　the reactions do not result in a large number of by-products that could interfere with the screening process, and so reactions that do not require extraneous reagents are preferred.

The reactions discussed above in relation to the 'in-situ'
15　synthesis would be particularly suitable, but other reactions could be considered, for example those using methodology in which the reagents that catalyse or drive the synthetic conversions are bound onto a solid phase medium and therefore removed from the solution by filtration. These chemistries, suitable for use with
20　this invention, are described by Ley, S.V., *et al.*, *Perkin 1*, 23, 3815-4195 (2000) and references cited therein.

Such reactions can be carried out using synthetic procedures described in general organic chemistry texts such as March's
25　Advanced Organic Chemistry (Smith, M.B. and March, J., 5th edition, Wiley-Interscience, New York, 2001) or references given therein, and reference is also made to the texts on the practice of combinatorial chemistry given above.

30　As in 'in-situ' synthesis, the reactions take place between appropriate sets of ligand precursor molecules ('monomers'), in which at least one substituent group is varied, to result in sets of monomers. Each reacting set of monomers can have as few as a single member, up to, for example, 40 members, although a maximum
35　of about 20 members would be more usual.　In some embodiments of the invention it is preferred that each set of reacting monomers comprises at least two monomers.　A typical size for the

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resulting collection of compounds would be between 5 and 1000,
preferably between 5 and 100, with a range 10 or 20 to 50 or 70
being preferred.

5   The usual purification and characterisation steps which are used
       in the practice of combinatorial or parallel synthesis are not
       required in methods according to the present invention. These
       steps are viewed as essential in the conventional practice of
       these synthesis methods in order to produce compounds suitable
10   for testing in a biological assay, as described in many of the
       references cited herein. Purification does not involve physical
       separation techniques such as solvent evaporation or removal of
       insolubles, e.g. by sedimentation, centrifugation or filtration.
       Conventional purification methods include aqueous extraction,
15   trituration, chromatography such as flash column chromatography
       or HPLC purification, crystallisation and distillation, although
       certain characterisation methods can also be used in
       purification. Characterisation may be carried out in a number of
       ways, including using LCMS, MS and NMR analysis.
20

       In a particularly preferred embodiment of the invention, the
       chemistry used for the 'just-in-time' synthesis is carried out in
       a solvent suitable as a co-solvent for aqueous solutions of
       protein crystals. Such solvents include DMSO, NMP and alcohols
25   such as methanol and ethanol (for more details, see discussion of
       cosolvents in 'in-situ' synthesis). In this way, after
       incubation of the reaction for a suitable period of time,
       aliquots can be taken of the solution and added directly to the
       protein crystal containing solution, without the need for any
30   purification or characterisation steps.

       Example
       The protein chosen as the target macromolecule was cyclin-
       dependent kinase 2 (CDK2). This target has been the subject of
35   intense study with the aim of developing inhibitors for the
       treatment of a number of human cancers, and has been crystallized
       with a number of inhibitors bound in the ATP-binding groove (De

Azevedo, W.F., et al., *Eur. J. Biochem.*, **243**, 518-526 (1997);
Hoessel, R., et al., *Nat. Cell Biol.*, **1**, 60-67 (1999))

   The collection of compounds chosen for synthesis were based on
5   the oxindole template, being a class of inhibitors already
   disclosed for CDK2 (Bramson, H.N., et al., *J. Med Chem.*, **44**,
   4339-4358 (2001)). These ligands (**AB**; Scheme 1) present
   substituents in adjacent lipophilic binding pockets within the
   ATP binding groove and can be disconnected to monomers of
10   approximately equal size and complexity (hydrazines **A** and isatins
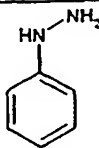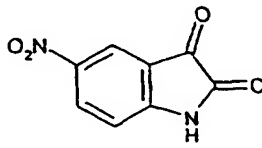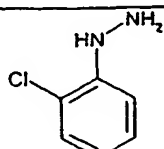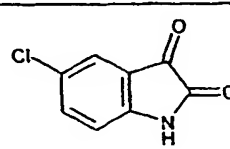   **B**; Scheme 1).

Scheme 1
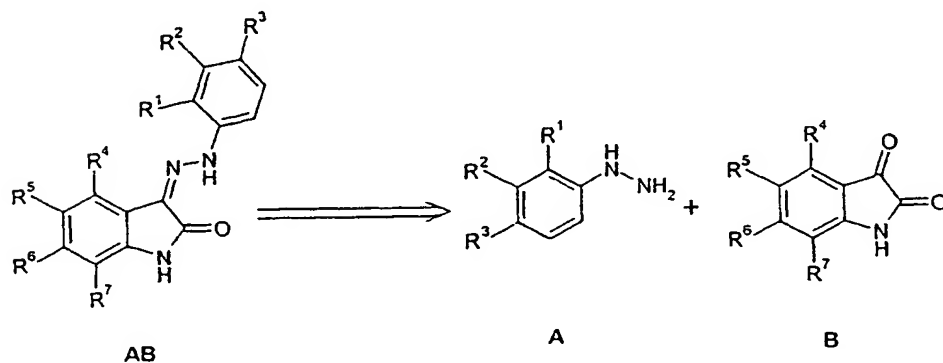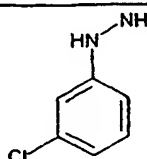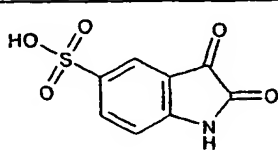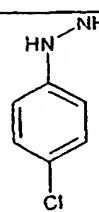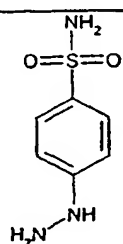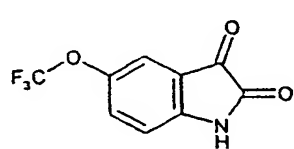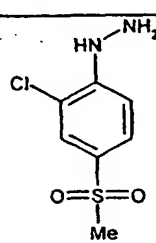
15

   A range of hydrazines (A1 to A6; Table 1) and isatins (B1 to B5;
   Table 1) were chosen, so that the collection of oxindoles formed
   would present a range of functional groups to the ATP binding
   site.

20

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Table 1

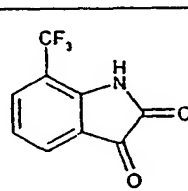| Hydrazines		Isatins	
A1		B1	
A2		B2	
A3		B3	
A4		B4	
A5		B5	
A6			

The synthesis of the collection of ligands was then demonstrated
to proceed under aqueous conditions in the presence of 20% of the
5   co-solvent dimethylsulfoxide (DMSO), according to scheme 2:

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Scheme 2

A   B   AB
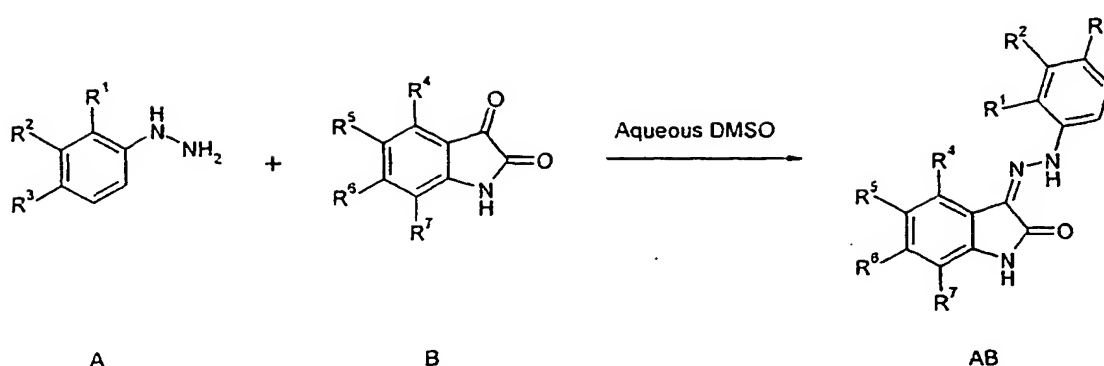
Analysis by mass spectrometry of the 30 reactions in the array
5   indicated that all reactions successfully formed the expected
products over a 24-72 hour period and the efficiency of the
individual reactions was assessed by LC/MS analysis (**Table 2**). In
most cases, under these reaction conditions, the products slowly
precipitated from the reaction solution over time. It is clear
10 from the qualitative data presented in Table 2 that monomer A6
did not give high conversions or high purity in the reactions and
that some other individual reactions were also poor, however, the
variability in absolute amount of products formed between
individual reactions would be expected to be no more than 10-fold
15 from these results.

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Table 2 (% purity by peak area of product by LC/MS)

A X B	A1	A2 R^1 = Cl	A3 R^2 = Cl	A4 R^3 = Cl	A5 R^3 = SO_2NH_2	A6 R^1 = Cl; R^3 = SO_2Me
B1 R^5 = NO_2	10-25	60-95	60-95	30-50	60-95	30-50
B2 R^5 = Cl	60-95	60-95	60-95	60-95	60-95	30-50
B3 R^5 = SO_3H	10-25	60-95	30-50	10-25	60-95	30-50
B4 R^7 = CF_3	30-50	60-95	60-95	60-95	60-95	30-50
B5 R^5 = OCF_3	30-50	60-95	60-95	60-95	60-95	10-25

R groups = H unless indicated otherwise.

Monomer studies to investigate kinetic competition of the
monomers indicated that the products formed non-stoichiometric
mixtures since it would be expected that the hydrazines and
isatins would have varying reactivities. LC/MS analysis of
competition experiments (using Photodioide Array Detector
scanning from 200-400 nm wavelengths) indicated that mixtures of
the hydrazines reacted with a 10-fold deficit of each isatin gave
less than a 5-fold excess of any individual product over any
other and typically less than 3-fold excess. These data again
indicated that monomer A6 tended to be disfavored but again a
measurable amount of product resulting from reaction of this
monomer with each isatin was detected (5-10% of the mixture).

These results show that during ligand synthesis in the presence
of the protein all of the reaction products were capable of being
formed at a useful concentration. Additionally, under these
conditions, thermodynamic products would be expected to

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predominate because the reactions are fully reversible.

Analysis of the reactions, e.g. by LC-MS and isolation of the
compounds is not required for the method of the invention, but
5   having now established reaction conditions suitable for use in
the X-ray screening method disclosed in this invention, it is now
possible to change the substitution patterns present in the
monomer sets A and B (i.e. R^1-R^7) and to carry out *in situ*
synthesis of further libraries of compounds, not described
10   herein, in the presence of CDK2 crystals.

Crystals of full length (residues 1-298), human, cdk2 were grown.
For soaking purposes crystals were transferred into a solution
that maintained the ionic strength and precipitant concentrations
15   of the original crystal mother liquor, but also contained 20%
DMSO and the hydrazine and isatin reactive species (see **Table 3**).
In all cases the total hydrazine concentration was in 10-fold
excess over the isatin concentration.

20   The present invention also includes the use of the aforementioned
methods for the generation of CDK2 ligands.

Experimental Methods
Crystals of full length (amino acids 1-298) human cyclin
25   dependent kinase 2 (cdk2) were grown under the conditions
detailed in (1) Lawrie, *et al.*, *Nat. Str. Biol.*, 4, 796-800
(1997) and (2) Rosenblatt, J., *et al.*, *J. Mol. Biol.*, 230, 1317-
1319 (1993). Crystals grown using (1) were obtained using the
hanging drop, vapour diffusion method, at 4°C, or 18°C. 1 μl of
30   10 mg/ml cdk2 in 10 mM Hepes/NaOH pH 7.4, 15 mM NaCl, was mixed
with 1 μl of reservoir solution. The reservoir solutions (1 ml
total volume) contained (25-55) mM ammonium acetate, (10-17.5)%
polyethylene glycol (PEG)(average molecular weight 3350) and 100
mM HEPES/NaOH pH 7.4. Crystals grown using (2) were also
35   produced by the hanging drop, vapour diffusion, method at 4°C. 4
μl of 10 mg/ml cdk2 in 10 mM HEPES/NaOH pH7.4 were suspended over

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a 1ml reservoir solution containing (200-800) mM HEPES/NaOH pH
7.4.

For soaking purposes crystals grown using (1) were transferred
into microbridges containing 20 µl of soak solution. Soak
solutions were prepared such that the ammonium acetate, PEG and
HEPES/NaOH pH 7.4 concentrations were identical to the drop from
which a given crystal was harvested. The soak solutions further
contained 20% DMSO and the hydrazine and isatin species that were
to be reacted. Hydrazine and isatin concentrations in the ranges
(5-20) mM (total organic) and (0.5-2) mM (total organic)
respectively were used. Total organic refers to the fact that in
a soak solution containing multiple isatins and hydrazines the
combined concentration of all the hydrazines would be (5-20) mM
and the combined concentration of all the isatins would be (0.5-2
mM). All hydrazines were present in equimolar concentrations and
all isatins were present in equimolar concentrations. That is;
if a soak solution contained two isatins and four hydrazines with
total organic hydrazine and isatin concentrations of 10 mM and 1
mM respectively each of the isatins would be present at a
concentration of 0.5 mM and each of the hydrazines at a
concentration of 2.5 mM. Soak solutions typically contained
permutations of (1-5) isatins and/or (1-6) hydrazines (see Table
1.). The generic formula for the products formed by permuting
the reactants is given in Scheme 2 (see also Table 2.). The
exact nature of the products formed is detailed in Table 2.
Crystals were soaked for 3-5 days at 18°C.

Crystals were frozen by momentarily dipping them into a cryo-
protectant solution and then snap cooling them in liquid
nitrogen. The cryo-protectant solutions contained (17.5-22.5)%
glycerol and ammonium acetate, PEG and HEPES/NaOH pH 7.4
concentrations that were identical to the drops from which the
crystals were originally harvested.

Crystals grown using (2) were soaked, and frozen identically to
those grown using (1), except that rather than maintaining

ammonium acetate, PEG and HEPES/NaOH pH 7.4 concentrations only
the HEPES/NaOH pH 7.4 concentration was maintained.

X-ray diffraction data were collected from soaked crystals,
5   cooled to 100K, on a Rigaku copper rotating anode source using
Rigaku/MSC Jupiter CCD, or Raxis IV++ image plate, detectors.
The data were integrated, reduced and scaled using either the
D*TREK suite (Pflugrath, J.W., *Acta Crystallographica*, D55, 1718-
1725 (1999)), or MOSFLM (Leslie, A.G.W. In *Joint CCP4 and EESF-*
10   *EACMB Newsletter on Protein Crystallography*, vol. 26, Warrington,
Daresbury Laboratory (1992)), SCALA and the CCP4 suite of
programs (CCP4: see above). An apo-cdk2 structure (DeBondt,
H.L., *et al.*, *Nature*, **363**, 595-602 (1993)) was used as a starting
point for structure refinements. The structures were initially
15   segmented into 25 amino acid sections and rigid-body refined in
CNX (see above). The structures were then subjected to iterative
cycles of positional and isotropic B-factor refinement in CNX ,
followed by manual rebuilding using the graphics program "O" (see
above) and automated ligand fitting using AUTOSOLVE®. Final
20   water fitting was performed using in-house software. Details of
representative X-ray data are given in Table 3.

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Table 3. Representative X-ray results and experimental conditions

Components in soaking solution	A5+B2	A5+B3	A5+B5	A5+B1	B2+ (A1,A2, A3,A4)	B2+ (A1,A2, A3,A4, A5,A6)	(B1,B2,B3, B4,B5)+ (A1,A2,A3, A4,A5,A6)
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
a (Å)	53.68	53.69	53.50	53.73	53.53	53.62	53.69
b (Å)	71.94	71.94	71.41	72.11	71.48	71.83	72.41
c (Å)	71.90	72.25	72.12	72.36	72.22	71.89	72.10
Maximal resolution	2.20	2.25	2.65	2.70	2.20	2.20	2.80
Observations	36740	41480	22249	23195	36709	35997	22680
Unique reflections	14265	13583	8277	8470	14171	14250	6991
Completeness (%)	97.2	98.5	98.2	98.6	92.7	89.1	96.9
R_{merge}	0.088	0.153	0.122	0.143	0.057	0.035	0.127
Mean I/σI	4.3	4.1	3.8	3.7	6.2	8.6	4.8
Highest resolution bin (Å)	2.28-2.2	2.33-2.25	2.74-2.65	2.74-2.65	2.28-2.2	2.28-2.2	2.95-2.8
Completeness (%)	88.5	97.8	99.5	100	86.8	85.2	98.0
R_{merge}	0.28	0.284	0.332	0.344	0.187	0.134	0.258
Mean I/σI	2.0	2.4	2.0	2.1	2.3	3.0	2.8
Refinement							
Protein atoms	2279	2279	2279	2279	2279	2279	2279
Other atoms							
Inhibitor	23	26	27	25	0	23	23
Water	139	129	57	39	245	229	67
Resolution range (Å)	35-2.2	35-2.25	35-2.65	35-2.7	35-2.2	35-2.2	35-2.8
R_{conv} (%)	25.9	24.7	23.7	24.3	25.6	24.6	21.1
R_{free} (%)	30.6	28.9	29.0	28.1	27.2	25.5	24.9
Mean B-factor (Å²)							
Protein	37.0	38.1	42.2	49.0	26.1	30.8	30.6
Ligand	31.2	37.3	47.0	59.7	NA	24.3	25.5
Solvent	47.2	42.8	35.5	50.4	34.2	41.5	34.8
Soaks							
Isatin conc (mM)	2[6]	0.5[6]	0.5[6]	1[6]	1	1	1 (total organic[4])
Hydrazine conc (mM)	20	5	5	10	10 (total organic[4])	10 (total organic[4])	10 (total organic[4])
Soak time (days)	5	3	3	3	3	3	3
Product present	YES[5]	YES[5]	YES[5]	YES[5]	NO[5]	YES[5]	YES[5]

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$^{1}R_{merge} = \Sigma_h \, \Sigma_j \, |I_{h,j} - \bar{I}_h| \, / \, \Sigma_h \, \Sigma_j \, |I_{h,j}|$, where $I_{h,j}$ is the jth observation of reflection h.

$^{2}R_{conv} = \Sigma_h \, ||F_o| - |F_c|| \, / \, \Sigma_h \, |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes respectively for the reflection h.

$^{3}R_{free}$ is equivalent to R_{conv}, but for a 5% subset of the reflections not used for refinement.

^{4} total organic refers to the total combined isatin, or hydrazine, concentration in the soak. Each component being present at a concentration equal to the total organic concentration divided by the number of isatin, or hydrazine, components in the mix.

^{5}Control experiments with crystals soaked for (3-5) days in cocktails containing either B2(20 mM), (B1,B2,B3,B4,B5)(1mM total organic^{4}), or (A1,A2,A3,A4,A5,A6)(1 mM total organic) did not reveal any interpretable ligand density. Crystals soaked in a 10 mM cocktail of (A1,A2,A3,A4,A5,A6) were repeatedly destroyed.

^{6}In certain instances it was found that crystals subjected to soaks that contained only one hydrazine and one isatin sustained increased damage as compared to those soaked in multi-component isatin-hydrazine cocktails. Isatin and hydrazine concentrations were iteratively reduced until soaking crystals could be stabilised, and crystal damage diminished. Precipitation, attributed to isatin-hydrazine product formation, was frequently observed during soaks.

Discussion of Results

Initial experiments were performed using reaction cocktails that contained a single isatin and a single hydrazine. In these instances, product binding was observed. Previous studies (Bramson, H.N., et al., J. Med Chem., 44, 4339-4358 (2001)) suggested that inhibitors derived from A5 (see Fig 1 & Table 2) should possess a relatively high degree of potency due to the presence of the sulphonamide group at the R^3 position in the ligand. A reaction cocktail containing B2 and (A1-A4) did not reveal ligand binding, suggesting that the chlorine substitutions at positions (R^1-R^3) did not confer significant potency upon the product ligands. A cocktail that contained B2+(A1-A6) did,

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however, reveal ligand binding; the electron density being
consistent with product formed by the reaction of (B2+A5). Thus
the (B2+A5) reaction product was preferentially selected from a
set of six possible products, lending support to the notion that
5   the R^3-sulphonamide group was conferring binding affinity upon the
A5B2 ligand. The degeneracy of the product library was increased
to 30 possible ligands using a reaction cocktail that contained
isatins (B1-B5) and hydrazines (A1-A6). Each isatin was present
at a concentration of 0.2 mM and each hydrazine at a
10   concentration of 1.67 mM (see Table 3). Difference electron
density consistent with the reaction product from (B2+A5) was
again observed. These studies suggest that the protein
preferentially selects the A5B2 ligand from the library of
available product ligands. The potency of this compound was
15   confirmed by synthesis of A5B2 ligand. It is known from the
literature that compound A5B2 has an IC50 of 43 nM (Bramson, et
al., 2001).